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PII: S0960-0760(19)30370-X

DOI: <https://doi.org/10.1016/j.jsbmb.2019.105536>

Reference: SBMB 105536

To appear in: *Journal of Steroid Biochemistry and Molecular Biology*

Received Date: 19 June 2019

Revised Date: 30 October 2019

Accepted Date: 6 November 2019

Please cite this article as: Rullo J, Pennimpede T, Far PM, Strube YN, Irrcher I, Urton T, Bona M, Gonder T, Campbell R, ten Hove M, Sharma S, Farmer J, Petkovich M, Intraocular Calcidiol: Uncovering a role for vitamin D in the eye, *Journal of Steroid Biochemistry and Molecular Biology* (2019), doi: <https://doi.org/10.1016/j.jsbmb.2019.105536>

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# Intraocular Calcidiol: Uncovering a role for vitamin D in the eye

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Word Count: 3990

## Highlights

- 25-hydroxyvitamin D<sub>3</sub> is minimally present in the aqueous humour of patients with cataract only disease
- Patients with various retinal diseases contain quantifiable 25-hydroxyvitamin D<sub>3</sub> in the aqueous and vitreous humour,
- The highest concentration of 25-hydroxyvitamin D<sub>3</sub> is found in patients with active neovascularization
- 1,25-dihydroxyvitamin D<sub>3</sub>-generating (*CYP27B1*) hydroxylases are present in the ciliary body and retina and correlate with *VEGF-A* expression.

## Abstract:

Vitamin D has emerged as a potentially important molecule in ophthalmology. To date, all ophthalmic data pertaining to vitamin D has been restricted primarily to tear and serum analysis in human patients. Considering the isolated nature of the eye, we sought to determine the presence of intraocular vitamin D in ocular disease.

Methods: 25-hydroxyvitamin D<sub>3</sub> (25(OH)D<sub>3</sub>) concentrations were measured in the eye and blood of 120 participants undergoing ophthalmic procedures. Ocular localization of the 1,25-dihydroxyvitamin D<sub>3</sub>-generating (*CYP27B1*) and deactivating (*CYP24A1*) hydroxylases was performed by immunohistochemistry. Gene expression of *CYP27B1*, *CYP24A1* and *VEGF-A* was measured in eyes from patients with and without disease.

Results: 25(OH)D<sub>3</sub> was quantified in 112 ocular samples. In 40 cataract patient samples, the average 25(OH)D<sub>3</sub> concentration was 0.057 ng/mL, compared to 72 retinal disease patient samples, average of 0.502 ng/mL ( $p < 0.001$ ). Intraocular 25(OH)D<sub>3</sub> did not correlate with serum levels of 25(OH)D<sub>3</sub>. There was no difference between the level

of 25(OH)D<sub>3</sub> measured in the aqueous and vitreous humour. The vitamin D-specific CYPs 27B1 and 24A1, strongly localized to complementary regions of the ciliary body, retinal pigment epithelium and neural retina. Gene expression analysis confirmed retinal *CYP27B1* correlated strongly with *VEGF-A* in eyes from diabetic patients ( $r=0.92$ ,  $p<0.001$ ).

**Conclusions:** Our data confirms that vitamin D is present in the humours of the human eye and that local synthesis/degradation is possible via the ocular CYP27B1 and CYP24A1. This argues for a functional role for local vitamin D production and signaling in the eye and suggests that vitamin D may be an important intraocular mediator in disease pathogenesis.

**Keywords:** ophthalmology, aqueous humour, vitreous humour, neovascularization, vascular endothelial growth factor, cataract surgery

**Abbreviations:** dry age-related macular degeneration (dAMD), non-proliferative diabetic retinopathy (NPDR), diabetic macular edema (DME), neovascular age-related macular degeneration (nvAMD), retinal vein occlusion (RVO), proliferative diabetic retinopathy/neovascular glaucoma (PDR/NVG), retinal detachment (RD), epiretinal membrane (ERM), and macular hole (MH). Intraocular pressure (IOP), vascular endothelial growth factor (VEGF).

## Introduction

The hormone vitamin D<sub>3</sub>, and its active derivative calcitriol (or 1,25 dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>) has received significant attention in the field of medicine for its pleotropic roles in preventing kidney disease, bone metabolism and immune modulation<sup>1,2</sup>.

Calcidiol or 25-hydroxyvitamin D<sub>3</sub> (25OHD<sub>3</sub>) is converted into the active 1,25(OH)<sub>2</sub>D<sub>3</sub> through a hydroxylation event catalyzed by the 1- $\alpha$  hydroxylase, or CYP27B1<sup>3</sup>. In clinical practice, 25(OH)D<sub>3</sub> is the standard metabolite that is measured, given its longer half-life and predominance in the circulation.

In the field of ophthalmology, interest in the so called “sunshine” vitamin has been gaining momentum for its deficiency being linked to cataracts<sup>4</sup>, myopia<sup>5</sup>, diabetic retinopathy<sup>6</sup>, uveitis<sup>7</sup>, glaucoma<sup>8</sup>, age-related macular degeneration<sup>9</sup>, retinopathy of prematurity<sup>10</sup> and optic neuritis<sup>11</sup>. Vitamin D has been hypothesized to play a protective role against the progression of age-related macular degeneration (AMD). Although initially not recognized as an inflammatory disease, it is now widely accepted that aberrant immune response including complement activation and the release of pro-inflammatory cytokines contributes to the pathogenesis of AMD<sup>12</sup>. Consistent with this, data from the third National Health and Nutrition Examination Survey showed that subjects with highest quintile of serum 25(OH)D<sub>3</sub> had a lower prevalence of early AMD and drusen formation compared to those in the lower quartile<sup>13</sup>. Moreover, single nucleotide polymorphisms in enzymes involved in the metabolism of vitamin D have been shown to predispose individuals to AMD<sup>14,15</sup>. Given its crucial role in regulating inflammatory processes, studies have examined vitamin D signaling in the context of uveitis. Anterior acute uveitis is the most common type of uveitis and is more prevalent

in patients with positive human leukocyte antigen (HLA)-B27. In a recent retrospective study, it was shown that polymorphisms in CYP27B1 are more common in HLA-B27 positive anterior uveitis patients compared to HLA-B27 positive controls without uveitis. Consequently, it is possible that dysfunctional vitamin D metabolism could create a more a pro-inflammatory environment in HLA-B27 positive individuals and further increases their risk of uveitis<sup>16</sup>. Vitamin D deficiency has also been linked to the presence and severity of diabetic retinopathy<sup>17</sup>. Further, the supplementation of 1,25-dihydroxyvitamin D to diabetic rats has been shown to be protective against glucose-mediated vascular damage via down regulation of various inflammatory pathways ultimately preventing damage to retinal vascular cells<sup>18</sup>.

Most of the evidence for vitamin D in the eye has been derived from in-vitro assays, in-vivo animal models, and the analysis of circulating calcidiol and calcitriol in the tears and serum<sup>19-21</sup>. Few studies have measured vitamin D and its metabolites in the aqueous and vitreous humour of patients. Taking into account the isolated and compartmentalized nature of the eye, with its well-defined blood-ocular barriers, the utility of analyzing serum vitamin D as a parameter in eye disease remains unfounded. Despite the numerous studies examining serum vitamin D in eye disease, the exact role of vitamin D within the humours (aqueous and vitreous) of the eye remains to be defined and even the existence of a functional role remains controversial. Considering the eye's unique anatomical position and constant exposure to ultraviolet light, we hypothesized that the eye contains vitamin D within ocular fluids, and possesses the required enzymes for its tissue-specific local metabolism. We set out to define the

intraocular status of vitamin D in patients with various ophthalmic disease conditions, as compared to the non-diseased state.

## **Methods**

### **Study Subjects**

We conducted a cross-sectional study of patients with a series of common eye disease to assess the levels of serum and intraocular vitamin D. This cross-sectional study was approved by the institutional ethics committee (Queen's University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board) and adhered to the tenets of the Declaration of Helsinki. Informed consent was obtained from all participants. Undiluted samples of ocular fluid (aqueous and vitreous humor) and blood serum were obtained from consented study participants undergoing surgical ophthalmic procedures from December 2016 to Dec 2018. Surgical procedures included anterior chamber paracentesis, pneumatic retinopexy, cataract extraction and pars plana vitrectomy. Primary participant diagnoses included cataract, retinal detachment (rhegmatogenous and tractional), neovascular age-related macular degeneration, diabetic macular edema, retinal vein occlusion, epiretinal membrane and macular hole. Exclusion criteria included active vitreous hemorrhage and patients undergoing dialysis or chemotherapy as these can alter aqueous humor dynamics. Detailed methodology is available in the supplemental materials.

### **Sample Collection**

Patients undergoing cataract surgery, intravitreal injection or pneumatic retinopexy received an anterior chamber paracentesis using a 1mL tuberculin syringe to remove 50-100uL of aqueous humour was removed. Vitreous humour was obtained during

standard 3-port pars plana vitrectomy. Paired serum samples were obtained from participants providing aqueous or vitreous humour samples. Samples were stored until further processing.

### **25-hydroxyvitamin D Detection**

In order to measure 25(OH)D<sub>3</sub>, a 50-100 µL sample of aqueous, vitreous or serum was sent to the Analytical Facility for Bioactive Molecules of The Centre for the Study of Complex Childhood Diseases, The Hospital for Sick Children (Toronto, Canada) for quantification of 25-hydroxyvitamin D. Full details are available in the supplemental methods section. Briefly, A 100 uL sample was extracted via liquid-liquid extraction using a Zinc Sulfate/Methanol/n-Hexane protocol by the Analytical Facility for Bioactive Molecules of The Centre for the Study of Complex Childhood Diseases. Samples were analysed by LCMS/MS using an Agilent 1290 HPLC interfaced with an AB Sciex 5500 Q-Trap mass spectrometer. Data were collected and analyzed using Sciex Analyst v1.6.3. The lower limit of quantification for 25(OH)D<sub>3</sub> was 0.200ng/mL. Quantified 25(OH)D<sub>3</sub> was tabulated according to group category as illustrated in the results. Samples below the limit of detection were given a value of 0.

### **Immuno-localization of CYP27B1 and CYP24A1**

The expression of calcitriol generating (CYP 27B1) and deactivating (CYP24A1) enzymes was detected using immunohistochemistry. Twenty-five formalin-fixed paraffin embedded healthy control eye sections (4-5µm sections) were obtained from The Human Eye Biobank (Toronto, Ontario). Sections were processed using standard methods, and incubated with either anti-CYP 27B1 (Abcam, Toronto, Canada), at a dilution of 1:2000 or anti-CYP 24A1 (Abcam), 1:400 dilution. The detection system



performed used alkaline phosphatase labelled anti-rabbit secondary to produce a red precipitate followed by hematoxylin counter staining (blue). A negative staining control with secondary antibody only and one with chromagen only were performed.

### **RNA isolation and qPCR**

Fresh whole eyes were obtained following cadaveric enucleation from the Canadian Eye Bank (Toronto, Ontario). Retinal tissue was dissected and total RNA isolated from ~100mg of retinal tissue using the PureLink™ RNA Mini Kit (ThermoFisher Scientific, Mississauga, ON) according to the manufacturer's instructions. Subsequent analysis of gene expression for *hCYP27B1*, *hCYP24A1*, *hIL-6*, *hCTGF*, *hTGF- $\beta$*  and *hVEGFA* was performed by qPCR on a ViiA7 Real Time PCR system using SYBR green detection (Power Up SYBR Mastermix, ThermoFisher). Mean gene of interest expression relative to reference gene, phosphomannomutase-2 (PMM2), was reported.

### **Statistics**

Descriptive statistics were used to evaluate frequencies and percentages for categorical data, and means, standard deviations, medians and quartiles for continuous data. Unpaired two-sided Student's t-test was used to compare quantitative data with a normal distribution. A p-value of 0.05 was used as the criteria for statistical significance. Statistical analysis was performed using Graphpad Prism (version 8.0).

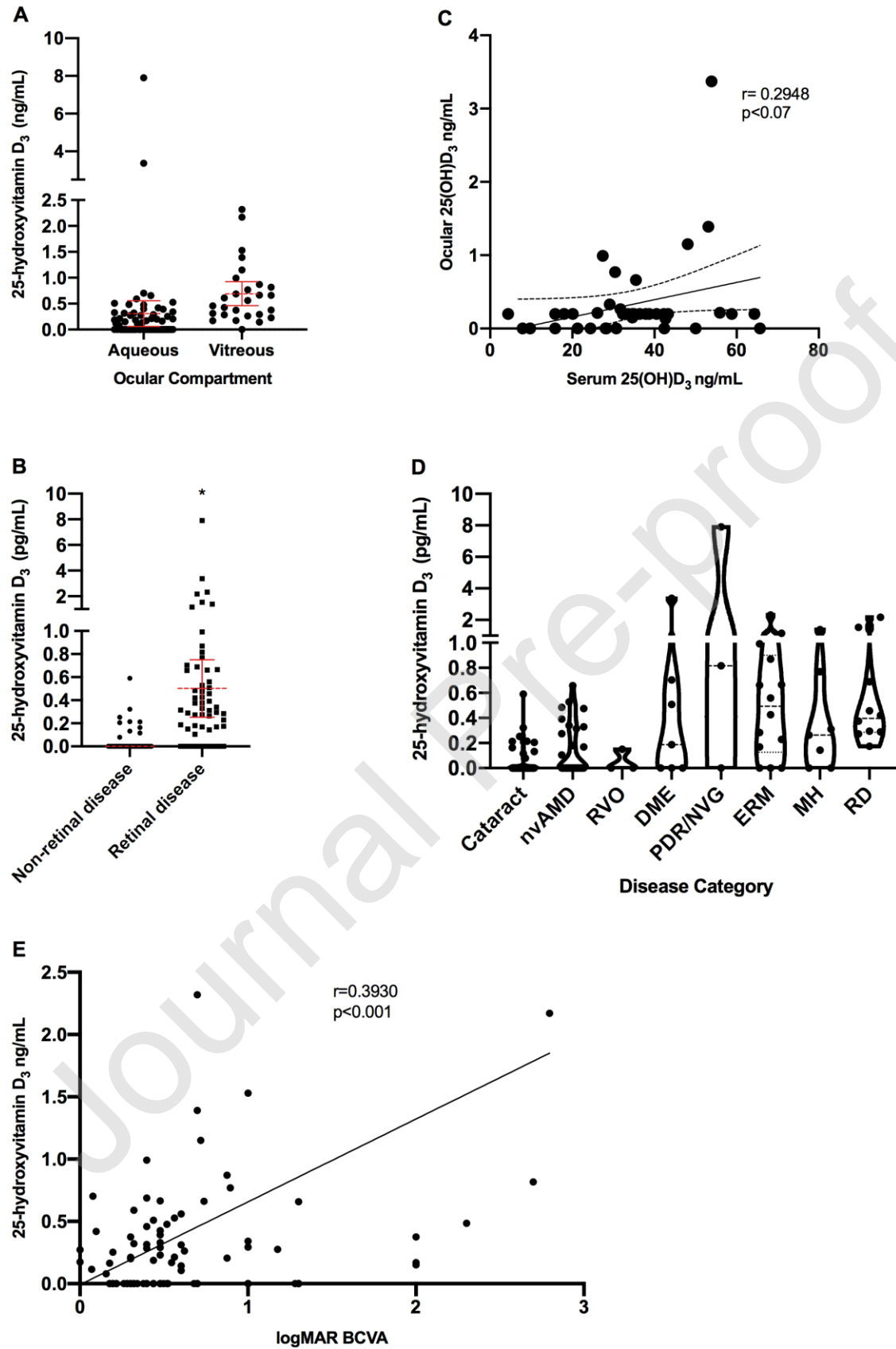
## Results

We obtained 86 aqueous and 34 vitreous samples from 117 participants. One hundred and twelve samples were available for analysis as three participants were excluded due to having undergone retinal dialysis or chemotherapy and two samples were of insufficient volume for mass spectrometry analysis. Forty samples were obtained from cataract surgery patients, 31 from vitrectomy patients and 41 from patients undergoing intravitreal injections. Paired ocular fluid and serum samples were available in 42 cases (34 aqueous samples, 8 vitreous samples). Patient disease categories included cataract, diabetic macular edema (DME), neovascular age-related macular degeneration (nvAMD), retinal vein occlusion (RVO), proliferative diabetic retinopathy/neovascular glaucoma (PDR/NVG), retinal detachment, epiretinal membrane (ERM), and macular hole (MH). Mean age of participants was 74 years (range 34-96 years), of which 54% of participants were male and 46% were female. Mean intraocular pressure (IOP) was 15.5 mmHg (range 7-34 mmHg). There was no difference in mean age among the three sampling groups. There was no correlation with ocular or serum vitamin D and age, gender or intraocular pressure (IOP). Table 1 displays participant baseline participant characteristics.

### Quantification of intraocular 25-Hydroxyvitamin D<sub>3</sub> in ocular disease

To determine whether vitamin D could be detected in the ocular fluid, 112 samples of aqueous or vitreous humor were analyzed by liquid chromatography- tandem mass spectrometry (LC-MS/MS) for the presence of 25(OH)D<sub>3</sub>. Quantifiable 25(OH)D<sub>3</sub> (above the limit of detection; >0.200ng/mL) in the aqueous and vitreous humour was 1 to 1.5

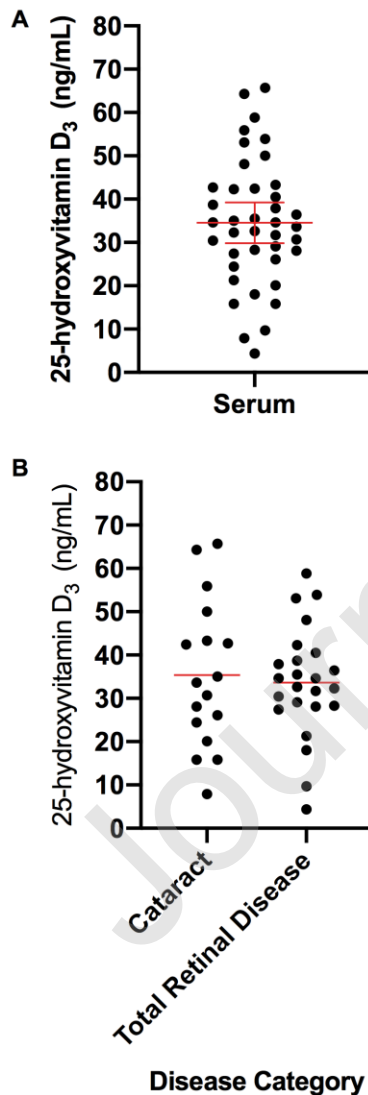
log lower compared to that in the serum (data not shown). Aqueous humour 25(OH)D<sub>3</sub> did not statistically differ from that of vitreous humour in all samples analyzed (Figure 1A). There was no significant Pearson's correlation observed between ocular fluid and serum 25(OH)D<sub>3</sub> (Figure 1B). 25(OH)D<sub>3</sub> was also compared in patients with and without active retinal disease. In 40 non-retinal disease cataract samples, the mean concentration of 25(OH)D<sub>3</sub> was  $0.057 \pm 0.121$  ng/mL compared to  $0.501 \pm 1.06$  ng/mL in 72 samples from participants with retinal disease (Figure 1C). Samples from each disease category were collated and plotted in Figure 1D to observe trends in 25(OH)D<sub>3</sub> levels. The mean concentration of 25(OH)D<sub>3</sub> in patients with neovascular age-related macular degeneration were  $0.157 \pm 0.208$  ng/mL. In diabetic retinopathy patients with active diabetic macular edema the concentration of 25(OH)D<sub>3</sub> was  $0.681 \pm 0.128$  ng/mL compared to  $2.91 \pm 4.35$  ng/mL in patients with proliferative diabetic retinopathy. Patients with retinal detachments, quantifiable 25(OH)D<sub>3</sub> in both the aqueous and vitreous was  $0.667 \pm 0.656$  ng/mL. Patients requiring vitrectomy for ERM or MH had measurable 25(OH)D<sub>3</sub> concentrations of  $0.596 \pm 0.622$  ng/mL and  $0.411 \pm 0.504$  ng/mL respectively. 25(OH)D<sub>3</sub> in the aqueous and vitreous humour significantly correlated with a worse participant logMAR best-corrected visual acuity (BCVA) taken at the time of sample removal (Figure 1E). There was no significant difference in the amount of 25(OH)D<sub>3</sub> comparing aqueous and vitreous humour of eyes with retinal disease (data not shown). The highest concentrations of 25(OH)D<sub>3</sub> were observed in 2 of 3 patients with active proliferative diabetic retinopathy with iris neovascularization, with individual values of 7.90 ng/mL and 3.37 ng/mL.



**Figure 1: Intraocular concentrations of 25(OH)D<sub>3</sub> in aqueous and vitreous humour from patients with several common ophthalmic diseases undergoing medical and surgical care.** Panel A displays the individual participant 25(OH)D<sub>3</sub> concentrations separated into aqueous and vitreous samples from 112 participants. No significant difference was calculated between aqueous and vitreous samples. In Panel B, Pearson's correlation was calculated between paired ocular fluid and serum samples. There was no statistically significant relationship observed between paired ocular fluid and serum samples. C) Measured 25(OH)D<sub>3</sub> concentration of all aqueous and vitreous non-retinal disease samples (40 samples from cataract participants) compared to retinal disease samples (72 samples from participants undergoing treatment for retinal disease). Unpaired two tailed Student's t-test performed between non-retinal and retinal disease group as indicated. \*-indicates  $p=0.0097$ . D) Violin box-plot graphing the median, 25<sup>th</sup> and 75<sup>th</sup> percentiles of 25(OH)D<sub>3</sub> concentrations from patients with cataract alone (N=40), neovascular age-related macular degeneration (nvAMD, N=27), diabetic macular edema (DME, N=7), retinal vein occlusion (RVO, N=3), retinal detachment (RD, N=10), proliferative diabetic retinopathy/neovascular glaucoma (PDR/NVG, n=3), epiretinal membrane (ERM, N=14) and macular hole (MH, N=7). Samples were not adequately powered to allow for statistical comparison. E) Pearson's correlation between ocular fluid 25(OH)D<sub>3</sub> concentrations and logMAR best-corrected visual acuity (BCVA) in 88 participants. Pearson's coefficient  $r=0.393$ , 95% confidence (0.200 to 0.557),  $p<0.001$ .

### **Serum 25-Hydroxyvitamin D and ocular disease**

In all 42 patients with serum vitamin D measurements, the mean 25(OH)D<sub>3</sub> serum level was 34.5±14.6 ng/mL (range 4.3 to 65.7 ng/mL) (Figure 2A). The mean 25(OH)D<sub>3</sub> serum level in the cataract only group was 35.4±16.9 ng/mL compared to 33.6±12.8 ng/mL for all patients with retinal disease (Figure 2B). The mean concentrations of serum 25(OH)D<sub>3</sub> according to disease category were as follows: DME 28.9 ng/mL, PDR 35.6 ng/mL, nvAMD 35.2 ng/mL and ERM 33.6 ng/mL. Mean levels of 25(OH)D<sub>3</sub> were not statistically different between groups (Figure 2B).



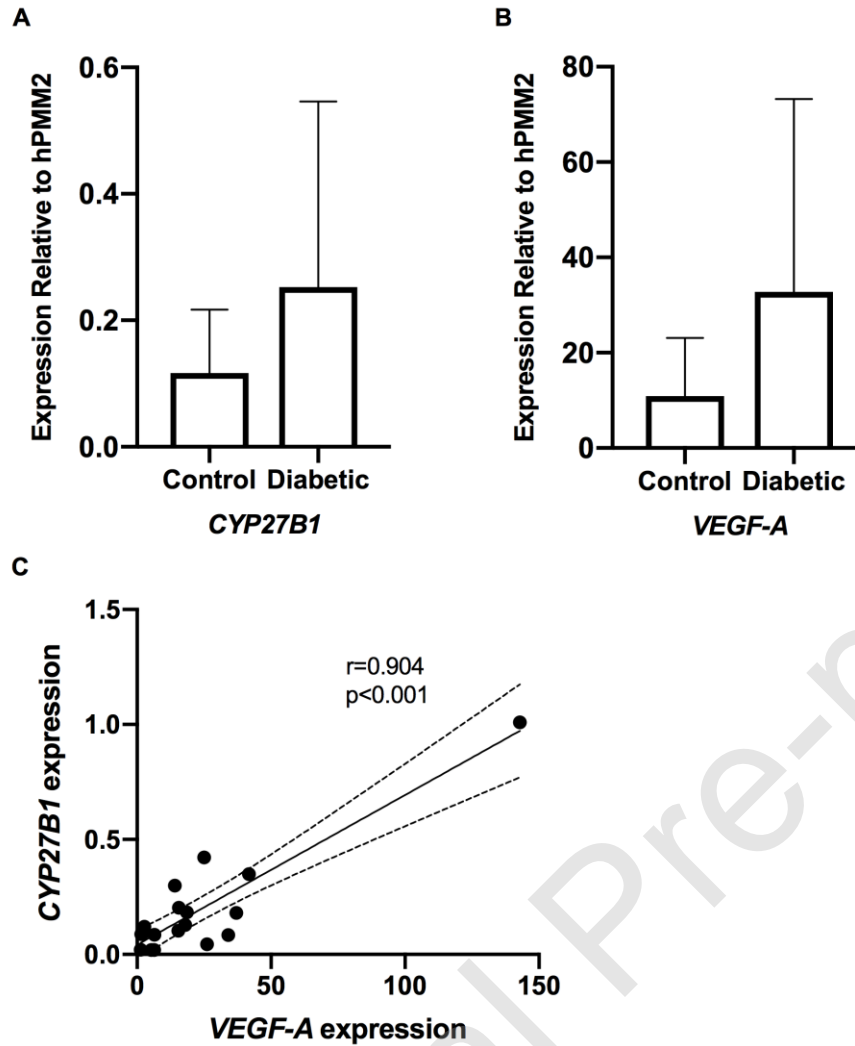
**Figure 2: Concentrations of serum 25(OH)D<sub>3</sub> from a medical and surgical ophthalmic population.**

In Panel A, a dot plot illustration of serum 25(OH)D<sub>3</sub> concentration from an ophthalmic population (N=42). Mean concentration with 95% confidence interval plotted in red.

Panel B shows a dot plot of serum 25(OH)D<sub>3</sub> comparing cataract patient samples from all retinal disease samples. The plot shows mean concentration (red line) of serum 25(OH)D<sub>3</sub> in the two subgroups (cataract, N=17; total retinal disease, N=24). There was no statistical difference between groups.

**CYP27B1 and CYP 24A1 enzyme expression in diabetic and control eyes**

Quantitative RT-PCR was performed on fresh human retina to examine the expression of inflammatory markers and vitamin D genes-of-interest in diabetic retinopathy. Eight control eyes from 4 patients (3 males, 1 female) without ocular disease (mean age 62) were compared to 10 case eyes from 5 patients (4 males, 1 female) with type 2 diabetes (mean age 63.5). Vascular endothelial growth factor A (*VEGF-A*) gene expression was quantified as a marker of retinal disease. Mean *CYP27B1* and *VEGF-A* expression were two to three-fold higher in eyes from diabetic patients as compared to controls (Figure 3A, 3B). There was a very strong correlation between retinal *CYP27B1* and *VEGF-A* in cases and controls,  $r=0.90$ ,  $p<0.001$  (Figure 3C). There was no correlation between *CYP27B1* and either *CYP24A1*, *IL-6*, *CTGF*, *TGF-B* or *CD14* (data not shown).

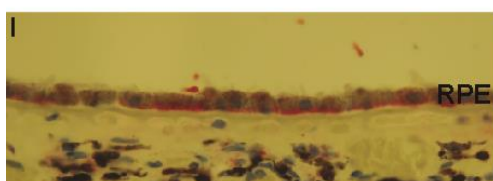
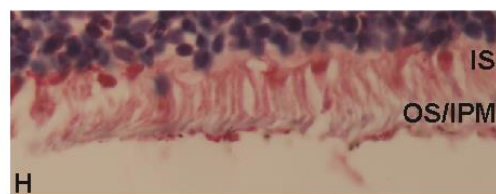
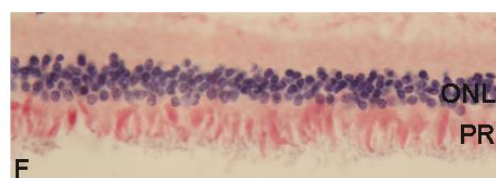
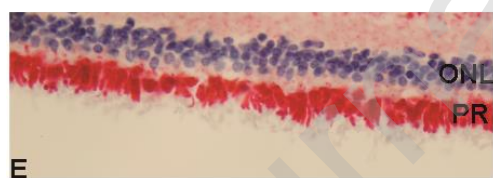
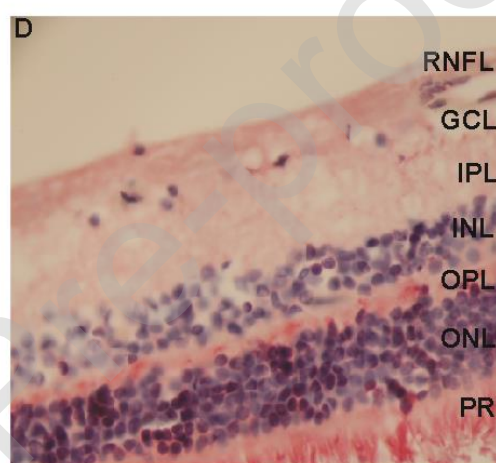
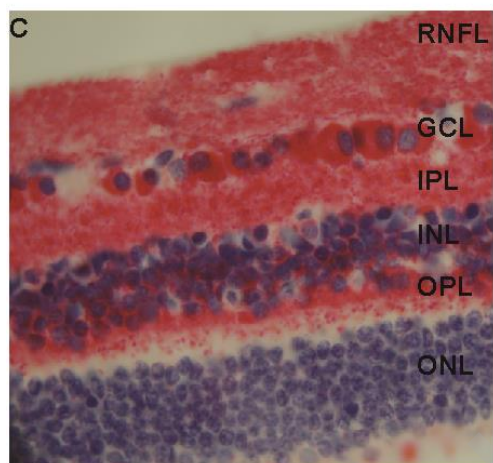
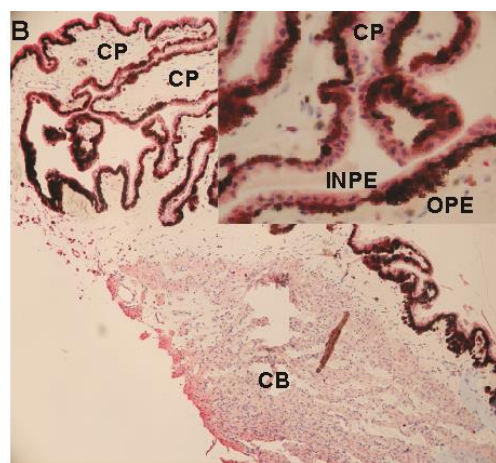
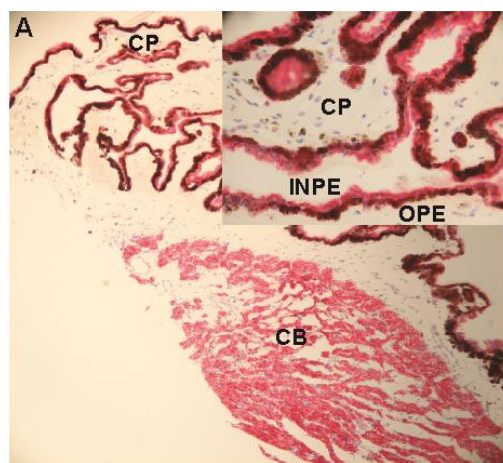


**Figure 3: mRNA expression of vitamin D activating (*CYP27B1*) gene and *VEGF-A* in control and diabetic eyes.** mRNA gene expression of *CYP27B1* (A) and *VEGF-A* (B) relative to phosphomannomutase-2 (PMM2). Control eyes, N=8, 4 patients. Diabetic eyes denoted, N=10, 5 patients. Mean gene of interest expression relative to PMM2  $\pm$  standard deviation B) Pearson's correlation between retinal *CYP27B1* and *VEGF-A* expression in control and diabetic eyes. Pearson's coefficient,  $r=0.904$ ,  $p<0.001$ , confidence interval (0.757 to 0.964).



### Immuno-localization of vitamin D enzymes in the eye

To determine the immuno-localization of the enzymes that convert  $25(\text{OH})\text{D}_3$  into active  $1,25(\text{OH})_2\text{D}_3$ , and the enzymes that catabolize active  $1,25(\text{OH})_2\text{D}_3$ , we performed immunohistochemistry for CYP27B1 and CYP24A1 on 25 cadaveric eye samples. All sections from 25 eyes showed a consistent and strong expression of CYP27B1 and CYP24A1 in the ciliary processes, ciliary body, neural retina, and retinal pigment epithelium. (Figure 4). CYP27B1 expression was inconsistently positive across samples in the basal layer of the corneal epithelium and endothelium (data not shown). CYP24A1 was not found to localize to any region of the cornea (data not shown). CYP27B1 was strongly expressed in both the inner non-pigmented and outer pigmented epithelial cells of the ciliary body in contrast to CYP24A1 which showed weak expression of the inner non-pigmented and strong expression of the outer pigmented epithelium (Figure 4 A,B). Neural retina expression of CYP27B1 showed strong expression throughout all the non-nuclear layers of the retina including the proximal but not distal (beyond the cilium) photoreceptor layer (Figure 4 C,E,G). CYP24A1 showed a weakly positive inner-, and strongly positive outer-retina staining, that being homogenous staining in the photoreceptor layer, extending into the interphotoreceptor matrix (Figure 4 E,F,H). Expression of CYP27B1 in the retinal pigment epithelium was restricted to the basolateral surface, whereas CYP24A1 had a more apical distribution (Figure 4 I,J).



**Figure 4: Immunolocalization of vitamin D activating (CYP27B1) and deactivating (CYP24A1) P450 enzymes.** Immunohistochemistry of retinal sections from cadaveric control eyes incubated with anti-CYP27B1 antibody (red), panels A, C, E, G, I or anti-CYP 24A1 antibody (red), panels B, D, F, H, J. Primary antibody localization denoted by red color. Counter-stain (blue) highlights nuclei (refer to methods for full details). A) CYP27B1 and B) CYP 24A1 localization within the ciliary body (CB) and extending ciliary processes (CP), original magnification 10x. High magnification insert (40X) illustrating the inner pigmented epithelium (INPE) and outer non-pigmented epithelium (ONP). C) CYP27B1 and D) CYP 24A1 localization of the inner and outer retina. Retinal nerve fibre layer (RNFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL) and photoreceptor layer (PR) highlighted. Original magnification 40x. E) CYP27B1 and F) CYP 24A1 localization of the outer retina only. Original magnification 20x. G) CYP27B1 and H) CYP 24A1 localization of the outer retina/retinal pigment epithelium (RPE) region. Inner segment (IS) of the photoreceptor, outersegment (OS) and interphotoreceptor matrix (IPM) highlighted. Original magnification 40X. I) CYP27B1 and J) CYP 24A1 localization of the RPE.

## Discussion

Here, we describe the quantifiable profile of intraocular 25(OH)D<sub>3</sub> in the aqueous and vitreous humour of patients with various common ophthalmic conditions as well as the expression pattern of vitamin D catalyzing enzymes in the eye. Active retinal neovascularization or damage to the neural retina is associated with the highest intraocular concentrations (aqueous or vitreous humour) of 25(OH)D<sub>3</sub>, compared to low or undetectable amounts in patients with only cataracts. Gene expression and immunolocalization confirmed the presence of the vitamin D catalyzing enzymes, CYP27B1 and CYP24A1 in the eye, with a predominance for the inner and outer retina (CYP27B1) and inner retina (CYP24A1), suggesting calcitriol is produced in the retina and able to signal to and within the RPE. Taken together, our data suggest a role for vitamin D in the eye, which appears to coincide with local accumulation in eye disease.

We measured the major circulating form of vitamin D, 25(OH)D<sub>3</sub> in the humors of the eye and serum along with detecting expression of the CYP family enzymes required for its conversion within the neural retina and ciliary body. Our data would infer that 1,25(OH)<sub>2</sub>D<sub>3</sub> could be locally produced within the eye. The absence of a correlation between aqueous or vitreous humour concentrations of 25(OH)D<sub>3</sub> and those in serum further suggests a locally-driven mechanism. This was most recently confirmed by Kim KL *et. al.*, measuring aqueous humour 25(OH)D<sub>3</sub> in diabetic macular edema patients compared to controls<sup>22</sup>. Here, aqueous humour 25(OH)D<sub>3</sub> concentrations was independent of the serum in both cases and controls<sup>22</sup>. A few additional studies have assessed the correlation between serum and ocular fluid levels of vitamin D to date.

Sethu *et. al.* found higher levels of 25(OH)D<sub>3</sub> in the tear film in comparison to serum levels, however, they found the two to be positively correlated<sup>23</sup>. On the other hand, in a recent report by Tsun Lai *et. al.*, no correlation between tear and blood levels of vitamin D was found as measured through electroluminescence<sup>24</sup>. The mechanism by which vitamin D circulates inside the eye compared to around may be mechanistically distinct as the tear constituents are related to a lacrimal gland functional unit where as aqueous humour is regulated by the tight junctions of the blood aqueous barrier and the functioning of the outer pigmented and inner non-pigmented epithelium of the ciliary body. Overall, there appears to be preliminary consensus in the literature, supported by our findings, that serum levels of vitamin D may not be a reliable measure of intraocular levels.

How vitamin D or its precursors enter the eye or whether it is produced locally remains experimental. In the skin, 7-dehydroxycholesterol (7-DHC), in the presence of UV-B light, is converted into a pre-vitamin D, which enters the liver for conversion into 25(OH)D<sub>3</sub><sup>25</sup>. UV-treated cell cultures of human ocular barrier cells is capable of producing active vitamin D, but only in the presence of exogenous 7-DHC<sup>26</sup>.

Considering the choroid of the eye receives the highest blood flow per gram of tissue in the body<sup>27</sup>, it is possible 25(OH)D<sub>3</sub> or its precursors, travel to the eye for conversion. However, the lack of a strong relationship between serum and ocular 25(OH)D<sub>3</sub> argues against this possibility. Yet, oral supplementation with vitamin D<sub>3</sub> (cholecalciferol) in rabbits is able to increase the amount of serum and aqueous humour concentrations of 25(OH)D<sub>3</sub><sup>26</sup>. Therefore, an actively conserved mechanism remains to be defined.

CYP27B1 along with other vitamin D<sub>3</sub> catalyzing enzymes have previously been detected at the mRNA level in human ocular barrier cell cultures and are functionally capable of converting vitamin D once inside the eye into its most active form<sup>20</sup>. Considering the in-situ expression of CYP27B1 and CYP24A1 in the ciliary body and epithelium (Figure 5), the site of aqueous humour generation, this would be a putative site for vitamin D precursors to enter and subsequently become converted prior to entering into the aqueous chamber. The expression of CYP27B1 within the neural retina implies 25(OH)D<sub>3</sub> is converted locally. The differential expression of CYP27B1 and CYP24A1 within the retina and RPE also highlights a topographical expression that may be important in normal retinal physiology. The location of CYP24A1 within the distal photoreceptor/interphotoreceptor matrix/apical RPE and CYP27A1 at the basolateral surface of the RPE could suggest local currents of vitamin D activation/deactivation important in photoreceptor function or outersegment disc phagocytosis. Considering the vitamin D receptor is expressed in photoreceptors<sup>28</sup>, the expression of these enzymes within the light-sensitive elements of the retina could suggest an unrecognized role for vitamin D in phototransduction. This remains highly speculative, but further research in the role for vitamin D in retinal neural physiology is now warranted.

Our data further supports the eye as one of the extra-renal organs with 1 $\alpha$ -hydroxylase activity, and therefore capable of local activation of 1,25(OH)<sub>2</sub>D<sub>3</sub>. This is supported by the presence of both substrate and enzyme in the ocular fluid and tissues respectively. Our research corroborates previous in-vitro cell culture studies using primary human scleral fibroblasts, corneal endothelial cells, ciliary body epithelium and retinal pigment



epithelium, all of which have been shown to produce 1,25(OH)<sub>2</sub>D<sub>3</sub><sup>20</sup> Ocular fluid 25(OH)D<sub>3</sub> may be analogous to 25(OH)D<sub>3</sub> in the serum, and therefore the ocular fluid acts as a conduit for transporting molecules important for dealing with chronic exposure to oxidative stress. Patients with cataracts contained the lowest concentrations of 25(OH)D<sub>3</sub>. Whether this is related to consumptive effect, a complete conversion into 1,25(OH)<sub>2</sub>D<sub>3</sub> or a reflection of a predominantly posterior eye segment phenomenon remains to be determined. Elevated concentrations of 25(OH)D<sub>3</sub> in the aqueous and vitreous humour could be related to an accumulation secondary to a breakdown in blood ocular barriers, especially considering the highest levels were seen in patients with proliferative diabetic retinopathy. Conversely, the elevated 25(OH)D<sub>3</sub> seen in patients with an ERM would argue against a breakdown in blood ocular barriers as the sole mechanism, but rather a more inflammatory or immune mediated phenomenon. Vitamin D has been shown to reduce the proliferation of CD8 T-cells, inactivate B cells and modulate the immune response towards a more T-regulatory dominant or immunosuppressive environment<sup>29-31</sup>. Considering the retinal predominant expression of CYP27B1, a rise in extracellular 25(OH)D<sub>3</sub> could reflect a backlog in substrate from the inability to convert into intracellular 1,25(OH)<sub>2</sub>D<sub>3</sub> as a result of enzymatic damage from various retinal disease stimuli. Alternatively, elevated 25(OH)D<sub>3</sub> may reflect an increased demand on the system, similar to what is seen in granulomatous disease. Unregulated production of 1,25(OH)<sub>2</sub>D<sub>3</sub> from extra-renal CYP27B1 in granulomatous macrophages is well established. Mycobacterium infection up regulates CYP27B1 and vitamin D receptor activity, which in the presence of exogenous 1,25(OH)<sub>2</sub>D<sub>3</sub> can activate antimicrobial molecules, such as cathelicidin, which in turn kills intracellular

mycobacterium<sup>32</sup>. The up-regulation of CYP27B1 and production of 1,25(OH)<sub>2</sub>D<sub>3</sub> also appears to be important in wound healing<sup>33</sup>. Therefore, measuring elevated 25(OH)D<sub>3</sub> in the ocular fluids secondary to widespread retinal injury is plausible. This may represent a new approach in understanding fundamental eye responses to disease.

In patients with retinal disease, the mean concentration of 25(OH)D<sub>3</sub> was 0.502 ng/mL as compared to 0.057 ng/mL in patients with cataracts only, a 9-fold magnitude difference. The highest concentrations were found in patients with active neovascularization, diabetic macular edema, epiretinal membrane formation or retinal detachment. The relative difference of 25(OH)D<sub>3</sub> in patients with retinal disease versus those without suggests vitamin D may be involved in the pathogenesis of many retinal diseases. Our data supports a growing body of research that highlights a new role for vitamin D in retinal vascular diseases. Our work also supports recent published work by Kim K.L. *et al.*, who showed elevated 25(OH)D<sub>3</sub> in the aqueous humour in patients with macular edema. A relationship between vitamin D signaling and VEGF-A in the context of neovascular diseases of the eye is intriguing. 1,25(OH)<sub>2</sub>D<sub>3</sub> present in smooth muscle cells is capable of driving VEGF expression through a vitamin D response element located in the VEGF promoter region<sup>34,35</sup>. Most recently, calcitriol engaging with the VDR in pericytes is able to increase the expression of VEGF, suggesting a pro-angiogenic capacity<sup>36</sup>. Calcitriol has also been shown to be capable of inhibiting neovascularization and reactive oxygen species in a mouse models of ophthalmic disease<sup>10,18,37</sup>. Therefore, 25(OH)D<sub>3</sub> measured in the humours of the eye may be elevated or driven by retinal neovascular disease activity. This is supported preliminarily



by the strong correlation observed between CYP27B1 and VEGF-A in diabetic eyes as well as the highest concentrations measured in patients with VEGF-dependent disease. Our work has a number of limitations. First, the cross-sectional nature inhibits investigators from drawing conclusions about the temporal relationship between ocular pathology and vitamin D regulation. Second, considering the sampling method for vitamin D, we are missing a critical control, namely health eyes without ophthalmic disease. The importance of this control cannot be overstated, but the ethical nature of sampling control eyes cannot be overlooked. The presence of vitamin D in the aqueous and vitreous humour in steady state conditions remains unknown. Future animal studies that recreate human retinal proliferative diseases will be essential. Third,  $1,25(\text{OH})_2\text{D}_3$  was not directly measured in this study and therefore we are not able to draw direct conclusions about ocular tissue levels of active vitamin D. The instability of  $1,25(\text{OH})_2\text{D}_3$  combined with the low volume of intraocular samples collected make measurement very difficult. Lastly, we do not know if there is a specific retinal-specific stimulus that drives  $25(\text{OH})\text{D}_3$  production, the conversion into  $1,25(\text{OH})_2\text{D}_3$  or if  $25(\text{OH})\text{D}_3$  is a by-product of local tissue damage and barrier disruption.

In conclusion, the relationship of ocular  $25(\text{OH})\text{D}_3$  with retinal abnormalities of the eye implicates vitamin D as a ocular disease target. If vitamin D enzymatic activity is up-regulated in retinal disease, local application of  $1,25(\text{OH})_2\text{D}_3$  may be therapeutic. Therefore, deciphering the ocular pathway of vitamin D will prove to be critical for understanding normal physiology and the pathophysiology of ocular disease. In the future, targeting vitamin D may be a new and exciting therapeutic approach to improving or preserving vision.

## Acknowledgements

We would like to thank Lee Boudreau from Queen's University, Department of Laboratory and Molecular Pathology for his contribution in the immunohistochemistry performed in this study.

The authors wish to thank Hayley Craig-Barnes, Ashley St. Pierre and Dr. Denis Reynaud of the Analytical Facility for Bioactive Molecules, The Hospital for Sick Children (Toronto, Canada) for assistance with vitamin D detection and measurement in the eye.

The work presented in this manuscript was in part funded by a grant from Barbera Tuck Macphee award from the CNIB to JR and through Emerging-Clinician Scientist Award from the Foundation Fighting Blindness Canada to JR.

Conflict of interest: Martin Petkovich is the chief scientific officer of Opko Renal, a division of Opko Health inc., which is involved in the treatment of chronic kidney disease using forms of Vitamin D3.

Sanjay Sharma is a speaker and advisor to research trials for Novartis and Bayer.

There was no industry input with respect to the design, participant recruitment, interpretation and analysis of data for this manuscript.

No other conflicting relationship exists for any other author.

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**Table 1: Baseline demographic participant characteristics**

<b>Demographics</b>			
Number of participants		117	
Number of intravitreal injection aqueous samples		43	
Number of cataract aqueous samples		41	
Number of vitrectomy vitreous samples		33	
Number samples excluded from participants		5	
<b>Age</b>	Mean, (SD)	74, (11)	
<b>Gender</b>		N, (%)	
	Male	63 (54)	
	Female	54 (46)	
<b>Mean intraocular pressure</b>	Mean, (SD)	15.5, (4.6)	
<b>Primary ocular disease</b>	Category	N, (%)	
<b>Total number of analyzed samples</b>		112	
	Cataract	40 (36)	
	nvAMD	27 (24)	
	DME	7 (6)	
	RVO	3 (3)	
	PDR/NVG	3 (3)	
	ERM	14 (12)	
	MH	7 (6)	
	RD	9 (8)	
dry age-related macular degeneration (dAMD), neovascular age-related macular degeneration (nvAMD)			
diabetic macular edema (DME), retinal vein occlusion (RVO), proliferative diabetic retinopathy (PDR)			
neovascular glaucoma (NVG), epiretinal membrane (ERM), macular hole (MH), retinal detachment (RD)			